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Salvianic acid A protects human neuroblastoma SH-SY5Y cells against MPP+-induced cytotoxicity

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Abstract

1-Methyl-4-phenylpyridinium ion (MPP+), an inhibitor of mitochondrial complex I, has been widely used as a neurotoxin because it elicits a severe Parkinson's disease-like syndrome with elevation of intracellular reactive oxygen species (ROS) level and apoptotic death. Salvianic acid A (SA), isolated from the Chinese herbal medicine *Salvia miltiorrhiza*, is capable of protecting diverse kinds of cells from damage caused by a variety of toxic stimuli. In the present study, we investigated the protective effects of SA on MPP+-induced cytotoxicity in human neuroblastoma SH-SY5Y cells, as well as the underlying mechanism. Treatment of SH-SY5Y cells with MPP+ caused the loss of cell viability, and condensation and fragmentation of nuclei, which was associated with the elevation of ROS level, the increase in Bax/Bcl-2 ratio, and the activation of caspase-3. MPP+ induced mitochondria dysfunction characterized by mitochondrial membrane potential loss and cytochrome *c* release. These phenotypes induced by MPP+ were reversed by SA. Our results suggested that the protective effects of SA on MPP+-induced cytotoxicity may be ascribed to its antioxidative properties and anti-apoptotic activity via regulating the expression of Bcl-2 and Bax. These data indicated that SA might provide a useful therapeutic strategy for the treatment of progressive neurodegenerative disease such as Parkinson's disease.
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Keywords: Salvianic acid A (SA); Antioxidation; Mitochondria; MPP+; SH-SY5Y cells; Bcl-2

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by the progressive loss of dopaminergic neurons in the substantia nigra. Even though the cause of Parkinson's disease remains to be answered, several lines of evidence strongly suggest the involvement of oxidative stress and mitochondrial dysfunction (Mattson, 2000). The major mitochondrial defect in Parkinson's disease appears to be associated with inhibition of respiratory chain complex I activity.

1-Methyl-4-phenylpyridinium (MPP+), the active metabolite of 1-methyl-4-phenyl-2,3,6-tetrahydropyridine (MPTP), has been shown to selectively and potently inhibit complex I of the mitochondrial electron transport chain (Singer and Ramsay, 1990) and induce a syndrome closely resembling Parkinson's disease in animal and cellular models (Przedborski and Jackson-Lewis, 1998), so it is usually used as the classic Parkinson's neurotoxin to study Parkinson's disease mechanism. It is thought that neuronal cell death induced by MPP+ is mediated by the opening of mitochondrial permeability transition (MPT) pore and the collapse of the mitochondrial membrane potential (Seaton wet al., 1997). While there is more than one pathway to apoptosis, the Bcl-2 family members play a significant role in MPP+-induced apoptotic cell death (Blum et al., 2001). O'Malley et al. (2003) reported that the overexpression of Bcl-2 attenuated MPP+-induced cell death. The interplay between pro- and anti-apoptotic Bcl-2 family members may determine the fate of cells by regulating the permeability of

mitochondrial membrane and controlling the release of cytochrome c from mitochondria (Yang et al., 1997; Cropton, 2000). Once released to the cytosol, cytochrome c could form apoptosome together with apoptosis-activating factor Apaf-1 and procaspase-9, leading to the activation of capase-9, and then activate capase-3 (Thornberry and Lazebnik, 1998). Caspase-3 has been demonstrated to participate in MPP+-induced apoptosis (Blum et al., 2001). Moreover, some previous works showed that reactive oxygen species (ROS) is also implicated in MPP+-induced cytotoxicity including mitochondrial permeability transition pore opening and cytochrome c release (Di Monte et al., 1986; Cassarino et al., 1999).

Salvia miltiorrhiza is a well-known Chinese traditional herbal medicine, which has been widely used in oriental medicine for treatment of heart and brain disease (Chen et al., 1979), hepatitis, hemorrhage, and menstrual abnormalities (Yasumasa et al., 1989). According to several phytochemical reports, salvianic acid A (SA) is one main effective component of Salvia miltiorrhiza. Previous studies showed that Salvia miltiorrhiza is an effective antioxidant (Zhao et al., 1996). SA could scavenge oxygen free radicals and inhibit lipid peroxidation (Zhao et al., 1996). It was reported that SA could exert a protective effect against cell death in experimental hepatitis (Li et al., 1996). Recently, it was suggested that the water-soluble extracts of Salvia miltiorrhiza could inhibit biliary obstruction-induced hepatocyte apoptosis by regulating the expression of Bax and Bcl-2 (Oh et al., 2002).

It is now believed that damage to dopaminergic neurons, involving oxidative stress and/or mitochondrial impairment, culminated in activation of an apoptotic cascade (Mattson, 2000). Regulation of intracellular ROS and modification of apoptotic cascades may control apoptotic events and provide new strategies for prevention and treatment Parkinson's disease. The purpose of the present study was to investigate the effects of SA on MPP+-induced cytotoxicity in human neuroblastoma SH-SY5Y cells in order to find a possible therapeutic application of the natural compound to degenerative diseases. In this study, we evaluated the protective effect of SA on SH-SY5Y cell against MPP+-induced cytotoxicity and demonstrated that SA inhibits cell viability loss and ROS elevation caused by MPP+. We also demonstrated that the alteration in the expression of Bcl-2 family leading to mitochondrial damage, cytochrome c release, and activation of caspase cascade, is involved in the regulation of MPP+-induced apoptosis by SA.

2. Materials and methods

2.1. Materials

Bax and Bcl-2 antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Cytochrome c antibodies were from Oncogene Research Products (San Diego, CA). 3-(4,5-Dimethyl-2-thiazolyl)-2,2,7-dichlorofluorescein diacetate (DCFH-DA), Hoechst 33258 and MPP+ were from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Gaithersburg, MD). The ApoAlert Caspase Fluorescent Assay kit was from Clontech (USA). Annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit was from Biosea BCL (China). SA (D(+)-β-3,4-dihydroxyphenol lactic acid), obtained from the National Medicine and Biological Product Inspection Institute of China, was dissolved in sterile double-distilled water at a concentration of 10 mg/ml and stored at $-20\,^{\circ}C$.

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum and 100 units/ml penicillin/streptomycin. Cells were kept at $37\,^{\circ}C$ in humidified 5% CO_2 and 95% air. All experiments were carried out 24–48 h after cells were seeded.

2.3. Number of viable cells

SH-SY5Y cells were incubated with different concentrations of SA in the presence or absence of 500 μM MPP+ for 24 h. The number of viable cells was determined with trypan blue exclusion assay (Jin et al., 2002). In brief, cells were collected and rinsed with phosphate-buffered saline (PBS). Then, cells were immediately stained with 0.4% trypan blue solution, and the number of viable cells was counted using a hemocytometer under a light microscope.

2.4. Nuclear staining with Hoechest 33258

After being treated with MPP+ and/or SA for 24 h, the cells incubated with DNA fluorochrome 3 μg/ml of Hoechst 33258 for 30 min. Then, cells were washed with PBS and analyzed by fluorescent microscopy (Olympus IX 71, Tokyo, Japan). Cells that exhibited reduced nuclear size, chromatin condensation, intense fluorescence, and nuclear fragmentation were considered to be apoptotic.

2.5. Measurement of intracellular reactive oxygen species

Intracellular ROS was monitored by using the fluorescent probe DCFH-DA (Lebel et al., 1990). Intracellular H_2O_2 or low-molecular-weight peroxides, oxidize DCFH-DA to the highly fluorescent compound dichlorofluorescein (DCF). SH-SY5Y cells were seeded in 96-well plates and were incubated with increasing concentrations of MPP+ and/or SA for 24 h. Cells were incubated with 10 μM DCFH-DA at $37\,^{\circ}C$ for 30 min, then washed twice with PBS, and finally the fluorescence intensity of DCF was measured in a microplate-reader (Fluoroskan Ascent Thermo Labsystems) at excitation wavelength 485 nm and emission wavelength 538 nm.

2.6. Immunoblot for Bcl-2 and Bax

After treatment, cells were collected and lysed as described previously (Hou et al., 2003). Cell lysate protein was loaded into a 12% SDS-polyacrylamide gel. After electrophoresis the gel was transferred to nitrocellulose membrane and blocked with non-fat milk in PBS containing Tween 20 prior to antibody treatments. After blocking, the blots were probed with anti-Bcl-2, anti-Bax antibody for 1 h, and further incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoblots were quantified by densitometric analysis (Chen and Chuang, 1999). Equal loading of protein in each lane was confirmed by probing with β-actin antibody.

2.7. Flow cytometric measurement of mitochondrial transmembrane potential

The level of mitochondrial transmembrane potential was determined by flow cytometry after being stained with rhodamine 123 (Emaus et al., 1986). After treatment, the cells were trypsinized, and washed twice with PBS. Five hundred microliters of cell suspension (1×10^6 cells/ml) were transferred to 5 ml culture tube and added rhodamine 123 solution at a final concentration of $1 \mu M$. After incubation for 30 min at $37\,^{\circ}C$, cells were analyzed using Becton Dickinson FACS Vantage flow cytometer. For each sample, 10000 events were collected.

2.8. Western blot assay for cytochrome c release

The release of mitochondrial cytochrome c was determined by Western blot. After treatment, cells were collected and washed with PBS, then resuspended in an ice-cold homogenizing buffer (250 mM sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin and 1 μg/ml leupeptin). Cells were homogenized with a glass dounce homogenizer. The homogenate was centrifuged at $1000 \times g$ for 10 min, and then resulting supernatant was subjected to $10,000 \times g$ centrifugation at $4\,^{\circ}C$ for 10 min. The supernatant was re-centrifuged at $10,000 \times g$ ($4\,^{\circ}C$, 1 h) to generate cytosol. Fifty micrograms proteins were subjected to SDS-polyacrylamide gel electrophoresis on a 12% gel. Then the protein in gel was transferred onto a nitrocellulose membrane and immunoblotted with cytochrome c antibody as described (Luo et al., 1998), finally incubated with goat anti-mouse IgG-horseradish peroxidase. The densitometric analysis of protein bands was performed by using image J analysis system.

2.9. Measurement of caspase-3 activity

Fluorometric assay of caspase-3 activity was conducted as described (Zeng et al., 2004). Briefly, after exposure to MPP$^+$ with or without SA treatment, cells were lysed for 10 min in an ice bath, and centrifuged at $14,000 \times g$ for 10 min at $4\,^{\circ}C$, then the supernatant were incubated with acetyl-Asp-Glu-Val-Asp-aldehyde-AFC, a pseudosubstrate used to measure caspase-3 activity, at $37\,^{\circ}C$ for 1 h. Fluorescence intensity was measured using F-4500 HITACHI fluorescence spectrophotometer (400 nm excitation and 505 nm emission).

2.10. Evaluation of apoptosis and necrosis

Both untreated and treated cells were harvested, washed and double-stained by using an annexin V–FITC apoptosis detection kit. This kit is based on the observation that soon after initiating apoptosis most cell types translocate the membrane phospholipid phosphatidylserin from the innerface of the plasma membrane to the cell surface (Vermes et al., 1995). Annexin V has a strong affinity for phospholipid phosphatidylserine and therefore serves as a probe for detecting apoptosis. Propidium iodide (PI) was added to cultured cells to identify the loss of integrity of the cell membrane which is specific for necrotic cells. Cells were incubated for 15 min in the dark with annexin V–FITC and PI, then were analyzed on the flow cytometer (Becton Dickinson FACS Vantage SE, USA). 10000 events were collected on each sample.

2.11. Statistical analysis

The data given in the text are expressed as mean $\pm$ S.E.M. The Student's t-test was used to analyze the relationship between the different variables. A difference was considered to be significant at $P < 0.05$.

3. Results

3.1. SA ameliorated MPP$^+$-induced loss of neuronal cell viability

In this study, the effect of SA on MPP$^+$-induced SH-SY5Y cell viability loss was assessed with trypan blue exclusion test. As shown in Fig. 1A, treatment with SA alone did not cause any cytotoxic effect on the cell viability up to the highest concentration (100 μg/ml). However, when the cells were treated with 500 μM MPP$^+$ for 24 h, their viability decreased to 63%, which was consistent with the previous results (Kakimura et al., 2001). The MPP$^+$-induced viability loss was attenuated by SA in a concentration-dependent manner. Treatment with 1, 10, 50, or 100 μg/ml of SA rescued the MPP$^+$-induced decease in viability rate by 3.7%, 9.8%, 18.2%, and 21.7%, respectively.

The involvement of ROS in the cytotoxic effect of MPP$^+$ was explored in the present study. As shown in Fig. 1B, antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) reduced the MPP$^+$-induced cell death, while the effect of 1 mM sulfhydryl reagent (dithiolthreitol) was not observed. The inhibitory effect of antioxidant on the
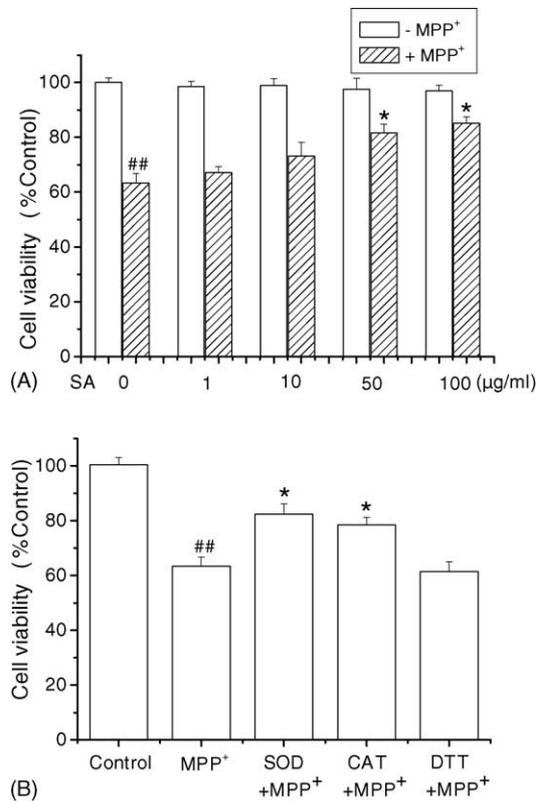
Fig. 1. Effect of SA on the MPP$^+$-induced decrease in SH-SY5Y cells viability. Cell viability was assessed by the trypan blue exclusion test as described in materials and methods. (A) Cells were treated with different concentrations of SA in the absence or presence of 500 μM MPP$^+$ for 24 h. (B) Cells were treated with 500 μM MPP$^+$ and/or 20 μg/ml SOD, or 20 μg/ml catalase (CAT), or 1 mM dithiolthreitol (DTT) for 24 h. Data are expressed as percentage of values in untreated control cultures, and are means ± S.E.M. ($n = 4$). $^*P < 0.05$, compared with the group treated by MPP$^+$ alone; $^{\#\#}P < 0.01$, compared with control (untreated group).

MPP$^+$-induced cell death suggested that the cytotoxic effect of MPP$^+$ may be mediated by oxidative stress.

3.2. SA rescued MPP$^+$-induced changes in nuclear morphology.

The nuclear morphological change was assessed using Hoechst 33258. As shown in Fig. 2, the normal SH-SY5Y cells nuclei had a regular and ovum shape. However, apoptotic nuclei characterized by nuclear condensation and fragmentation, appeared after exposure to 500 μM MPP$^+$ for 24 h. SA (50 μg/ml) treatment blocked the MPP$^+$-induced nuclear damage, while the nuclear morphology of cells exposed to SA alone was similar to that of untreated cells.

3.3. SA reduced MPP$^+$-induced increase of ROS in SH-SY5Y cell

MPP$^+$ interacts with mitochondrial complex I, blocking ATP production and promoting oxygen free radical formation (Adams et al., 1993). In this study, the degree

of ROS accumulation after MPP$^+$ exposure was measured to determine the role of ROS in MPP$^+$-induced neurotoxicity. As shown in Fig. 3, treatment of SH-SY5Y cells with MPP$^+$ (100, 200, and 500 μM) caused the increase of DCF fluorescence in a concentration-dependent manner, and increased by 1.33-, 1.67-, and 1.89-fold relative to control, respectively. However, simultaneous treatment with SA effectively reduced ROS generation, and the suppressing effect strengthened with the increase of the concentration of SA. SA (1, 10, 50, and 100 μg/ml) decreased 500 μM MPP$^+$-induced ROS production by 14%, 43%, 71%, and 86%, respectively. The results suggested the involvement of ROS in MPP$^+$-induced cytotoxicity and antioxidative activity of SA.

3.4. SA regulated the expression of Bcl-2 and Bax in MPP$^+$-treated cells

The Bcl-2 family consists of both apoptotic and anti-apoptotic proteins and the balance between these proteins is critical to turning on and off the cellular apoptotic machinery (Cory and Adams, 2002). Any shift in the balance of pro- and anti-apoptotic member will affect cell death. Bcl-2 family members are intimately involved in cell death processes caused by MPP$^+$ (O'Malley et al., 2003). Bcl-2 is an anti-apoptotic protein whereas Bax is pro-apoptotic (Cory and Adams, 2002). In this study, we investigated whether SA has any effect on the expressions of Bax and Bcl-2 in MPP$^+$-treated cells using Western blot analysis. As shown in Fig. 4A, Bax protein expressions increased significantly in 500 μM MPP$^+$-treated group compared with that in control. However, SA treatment (50 μg/ml) could decrease the Bax expression level almost to the normal values. Interestingly, the level of Bcl-2, which remained almost same in MPP$^+$ group as in control, increased upon SA treatment. The Bax/Bcl-2 ratio increased to 1.6-fold of control upon treatment with MPP$^+$, while SA prevented the MPP$^+$-induced increase of the Bax/Bcl-2 ratio (Fig. 4B). SA treatment alone did not significantly alter the Bax/Bcl-2 ratio. These results suggested a notion that SA treatment shifted the balance between positive and negative regulators of apoptosis towards cell survival.

3.5. SA blocked MPP$^+$-induced reduction of mitochondrial membrane potential

The Bcl-2 family has a regulatory role in mitochondrial permeability transition pore opening (Cropton, 2000). Upon mitochondrial permeability transition pore opening, mitochondria lose its membrane potential across the inner membrane. Disruption of the mitochondrial membrane potential has been recognized to be implicated in MPP$^+$-induced apoptosis (Seaton et al., 1997). To investigate the effect of SA on MPP$^+$-induced mitochondrial membrane potential reduction in SH-SY5Y cells, rhodamine 123 was used as a molecular probe to assay mitochondrial membrane
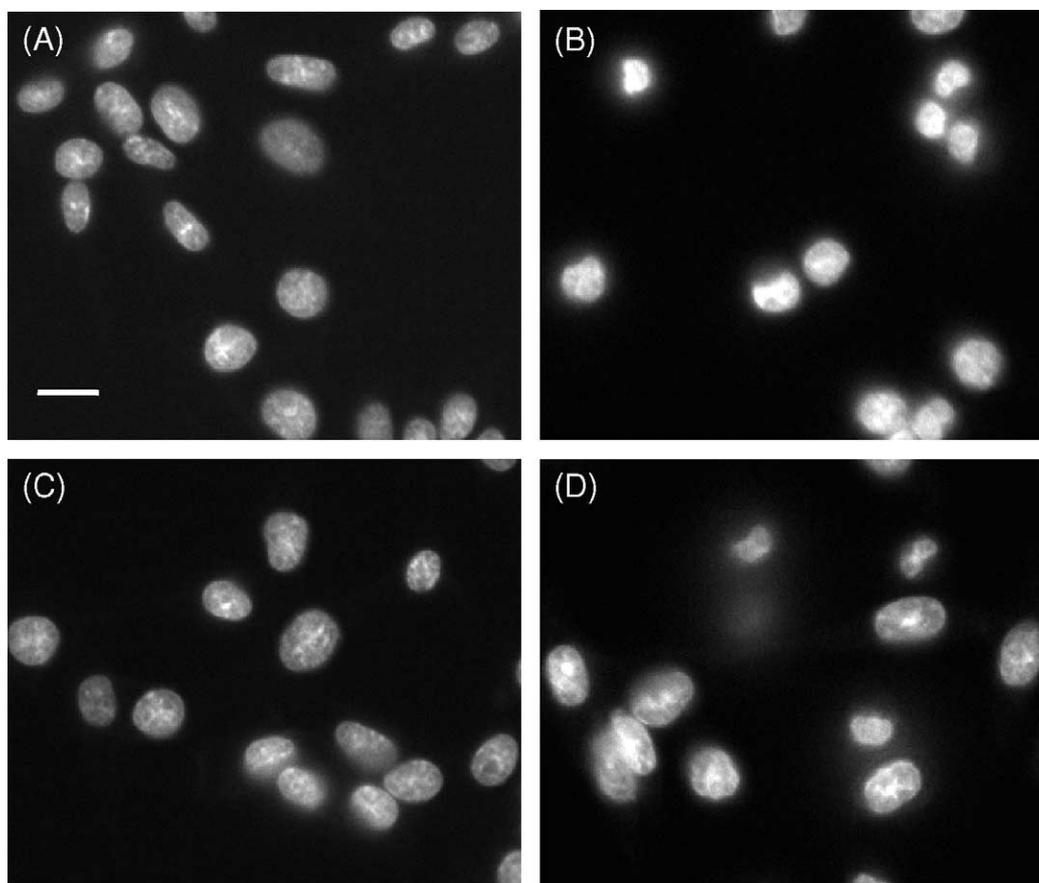
Fig. 2. Nuclear morphological assessment of SH-SY5Y cells by fluorescence microscopy. The figures show the fluorescence micrographs of Hoechst 33258 stained nuclear morphology. SH-SY5Y cells were untreated (A), treated with 500 μM MPP$^+$ for 24 h (B), treated with 50 μg/ml SA alone (C) or SA plus MPP$^+$ (D) for 24 h. Scale bar indicates 10 μM.

potential. The decrease of rhodamine 123 fluorescence intensity indicated the loss of mitochondrial membrane potential. As shown in Fig. 5A, the histograms exhibited that MPP$^+$ significantly caused collapse of mitochondrial
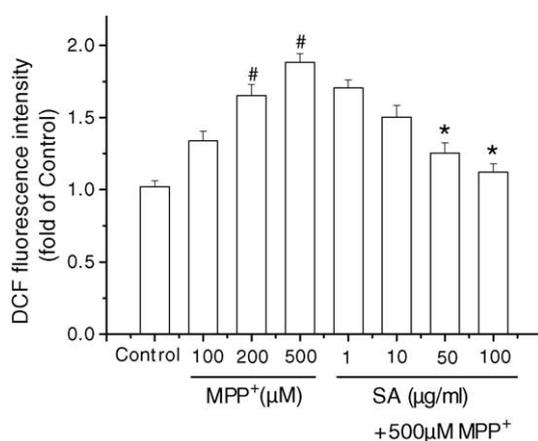

Fig. 3. SA reduced MPP$^+$-induced accumulation of ROS. The fluorescence intensity of DCF was measured after SH-SY5Y cells were exposed to MPP$^+$ and/or SA at the indicated concentration for 24 h. Data are expressed as means ± S.E.M. ($n = 6$). $^*P < 0.05$, compared with the group treated by 500 μM MPP$^+$ alone; $^\#P < 0.05$, compared with control.

membrane potential as evidenced by a shift to the left in fluorescence intensity. Fig. 5B revealed that SA prevented the MPP$^+$-induced increase in the percentage of cells with lower mitochondrial membrane potential. 10, 50, and 100 μg/ml of SA could decrease the percentage of cells with lower mitochondrial membrane potential caused by MPP$^+$ from 36.6% to 28.1%, 19.2%, and 14.1%, respectively.

3.6. SA inhibited MPP$^+$-induced cytochrome c release

The mitochondrial permeability transition pore opening is associated with collapse of the membrane voltage (Seaton et al., 1997), resulting in the release of cytochrome c into the cytosol (Nicholls and Budd, 2000). Cytochrome c release was proved to play a critical role in cell apoptosis (Kluck et al., 1997). The previous studies demonstrated that the MPP$^+$ could induce cytochrome c release from mitochondria to the cytosol (Cassarino et al., 1999). Using Western blotting, we investigated the possible effect of SA on the MPP$^+$-induced cytochrome c release from mitochondria. As shown in Fig. 6, 500 μM MPP$^+$ could induce cytochrome c release significantly, which is about 7-fold compared with that of control. However, the induction was markedly inhibited in
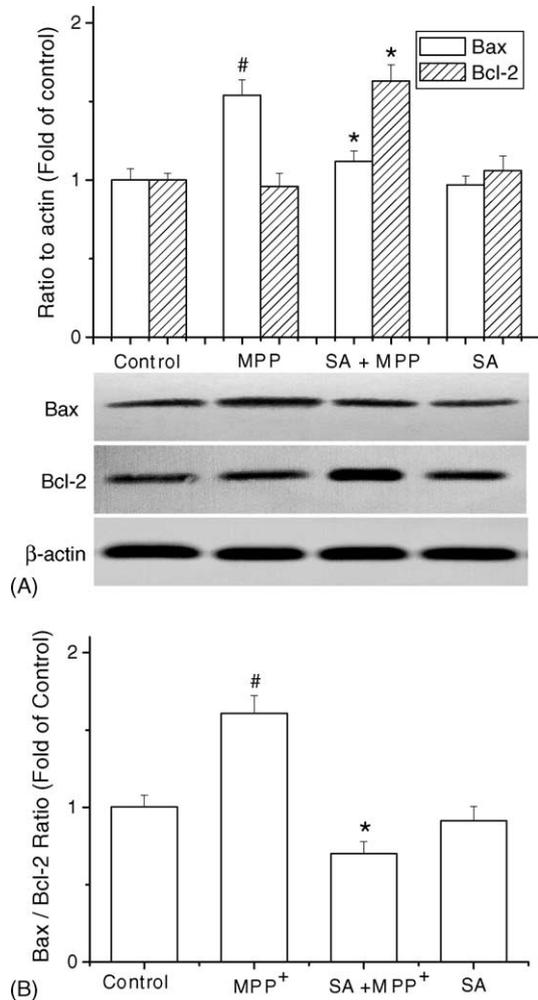
Fig. 4. Effect of SA on the expression of Bcl-2 and Bax in SH-SY5Y cells. Cells were treated with MPP[+] (500 μM) and/or SA (50 μg/ml) for 24 h, and then cell lysate were subject to Western blot analysis. The levels of Bax and Bcl-2 were quantified by densitometric analysis (A) and the Bax/Bcl-2 ratio was determined (B). Data are means ± S.E.M. ($n = 3$). $^{*}P < 0.05$, compared with MPP[+] alone; $^{\#}P < 0.05$, compared with control.

the presence of SA. The release of cytochrome c was inhibited 23% when cells were treated with 500 μM MPP[+] plus 10 μg/ml SA, and the inhibitory percentage was 51% when SA increased to 50 μg/ml.

3.7. SA suppressed MPP[+]-induced caspase-3 activity

Caspases are the molecular machinery that drives apoptosis (Markus, 2000). As caspase-3 is an important biomarker of the apoptosis process (Hartmann et al., 2000), its activity was examined in this study. Acetyl-Asp-Glu-Val-Asp-aldehyde-AFC, a pseudosubstrate, was used to measure caspase-3 activity. In the presence of caspase-3, this substrate is cleaved to the fluorochrome 7-amino-4-trifluoromethyl coumarin (AFC), which is quantified by measuring fluorescence intensity. The effect of SA on MPP[+]-induced caspase-3 activation is shown in Fig. 7. Following 24 h treatment of SH-SY5Y cells with MPP[+]
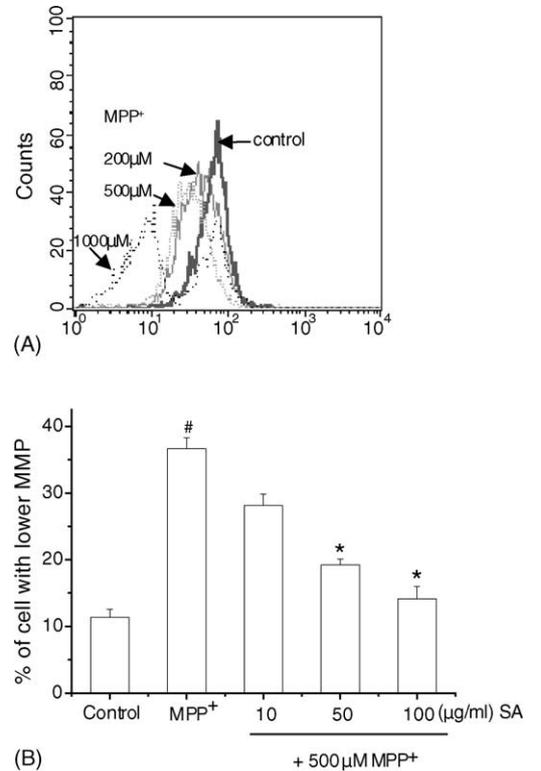

Fig. 5. Effect of SA on MPP[+]-induced mitochondrial membrane potential alteration. SH-SY5Y cells were exposed to MPP[+] and/or SA for 6 h, then mitochondrial membrane potential alteration was measured by flow cytometry using rhodamine 123 (Rh123) staining. (A) MPP[+] caused mitochondrial membrane potential loss. The histograms are one representative set of results from three independent experiments. (B) SA blocked MPP[+]-induced mitochondrial membrane potential loss. The percentage of cells with lower mitochondrial membrane potential (MMP) was calculated. Data are means ± S.E.M. ($n = 3$). $^{*}P < 0.05$, compared with MPP[+] alone; $^{\#}P < 0.05$, compared with control.

(500 μM), we detected a caspase-3 activity increase to 243% of the control level. The increase in caspase-3 activity was depressed by 20 μg/ml SOD and 20 μg/ml catalase.
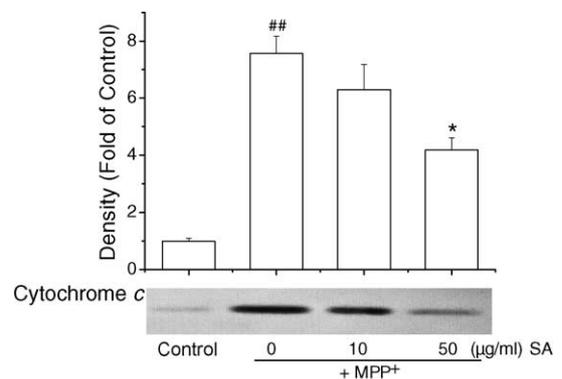
Fig. 6. SA blocked MPP[+]-induced cytochrome c release. SH-SY5Y cell were treated with 500 μM MPP[+], in the absence or presence of SA for 6 h. Cytochrome c release was determined by Western blot analysis. The amount of cytochrome c was estimated by densitometric analysis of each protein band. Data are means ± S.E.M. ($n = 3$). $^{*}P < 0.05$, compared with MPP[+] alone; $^{\#\#}P < 0.01$, compared with control.
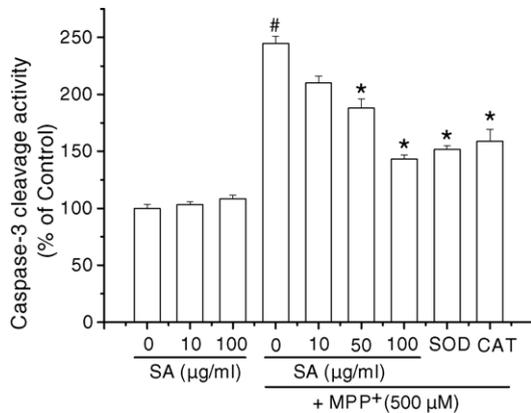
Fig. 7. SA inhibited MPP[+]-induced increase in caspase-3 activity. SH-SY5Y cells were treated with MPP[+] (500 μM) for 24 h in the presence or absence of SA, 20 μg/ml SOD, or 20 μg/ml catalase (CAT). Data are means ± S.E.M. ($n = 3$). [*]$P < 0.05$, compared with the group treated by MPP[+] alone; [#]$P < 0.05$, compared with control (untreated cells).

Addition of SA (10, 50, and 100 μg/ml) attenuated MPP[+]-induced caspase-3 activation and provided 23%, 41%, and 57% protection, respectively. SA alone did not show a significant effect on the caspase-3 activity in SH-SY5Y cells, which was consistent with its lack of apoptotic response (Fig. 2C).

3.8. SA attenuated MPP[+]-induced apoptosis

To further investigate whether the addition of SA affected MPP[+]-induced SH-SY5Y cell apoptosis, a flow cytometric
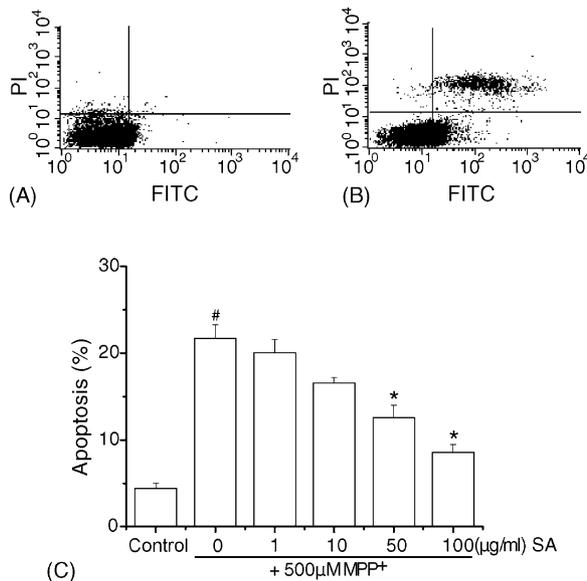

Fig. 8. Effect of SA on MPP[+]-induced apoptosis in SH-SY5Y cells measured by flow cytometry. SH-SY5Y cells were harvested and labeled with a combination of annexin V–FITC and PI, and analyzed by flow cytometer. The figure showed representative flow cytometric histograms of untreated cells (A) and cells exposed to 500 μM MPP[+] for 24 h (B). (C) Cells were treated with MPP[+] (500 μM) and/or SA for 24 h. Data are means ± S.E.M. ($n = 3$). [*]$P < 0.05$, compared with MPP[+] alone; [#]$P < 0.05$, compared with control.

analysis of phospholipids phosphatidylserine exposure on early apoptosis was conducted. Fig. 8A and B showed a display of PI versus annexin V–FITC fluorescence. The lower left quadrants of the cytograms show the viable (intact) cells, which exclude PI and are negative for annexin V–FITC binding. The lower right quadrants represent the apoptotic cells, which are annexin V–FITC positive and PI negative. The upper right quadrants contain necrotic cells, which are positive for annexin V binding and for PI uptake. In the control (Fig. 8A), 93.4% cells excluded PI and were negative for annexin V–FITC binding, which is viable cells. After exposed to 500 μM MPP[+] for 24 h, 33.4% cells showed annexin V positive (Fig. 8B), including 21.6% PI negative and 11.8% PI positive, which indicated apoptosis and necrosis, respectively. Addition of 1, 10, 50, and 100 μg/ml SA reduced the number of apoptotic cells evoked by MPP[+] (Fig. 8C). The percentage of apoptosis was decreased to 20.3%, 17.1%, 12.5%, and 8.7%, respectively.

4. Discussion

MPP[+]-treated human neuroblastoma SH-SY5Y cells are a useful model in vitro for studying neurodegenerative events that may occur in Parkinson's disease. The aim of the present investigation was to evaluate the protective effect and to determine the possible molecular mechanisms of SA on cytotoxicity induced by MPP[+] in SH-SY5Y cells. In this study, we demonstrated that SA protects SH-SY5Y cells against MPP[+]-induced cytotoxicity in several aspects. SA ameliorates MPP[+]-induced ROS production, increases the number of viable cells, inhibits apoptotic pathways, prevents caspase-3 activation, and decreases the number of apoptotic cells. Up to date, the cellular and molecular mechanisms that underlie the action of SA have not fully been understood. Here, our results demonstrated that several mechanisms, separately or in association, may be involved in the neuroprotective effects of SA.

The antioxidant effect is a possible mechanism for SA-mediated neuroprotection. Previous data demonstrated that oxidative damage occurs in Parkinsonian brain (Mattson, 2000). Overproduction of ROS can cause severe impairment of cellular functions. For instance, it can peroxidize membrane lipids (Butterfield et al., 1997) and oxidize protein, attack cytoplasmic RNA and mitochondrial DNA (Mecocci et al., 1994; Nunomura et al., 1999). Several studies have demonstrated that ROS are involved in the apoptotic mechanism of MPP[+]-mediated neurotoxicity (Di Monte et al., 1986) and may contribute to the apoptotic processes found in Parkinson's disease (Kehrer and Smith, 1994). Oxidative stress generated by MPP[+] might be, at least in part, responsible for the opening of mitochondrial permeability transition pore and the collapse of mitochondrial membrane potential (Cassarino et al., 1999). As mentioned previously, data from this study also show that treatment with MPP[+] results in a significant increase of ROS (Fig. 3). To determine whether suppression of ROS production

was effective to prevent apoptosis, we employed antioxidant enzyme (SOD and catalase) to examine their effects on SH-SY5Y cell death induced by MPP$^+$. Our results showed that SOD and catalase suppressed MPP$^+$-induced cell death and caspase-3 activation. The depressant effects of antioxidant enzymes, suggest the involvement of ROS in the cytotoxic effect of MPP$^+$ on SH-SY5Y cells. However, the present study also revealed that sulfhydryl reagent (dithiothreitol) did not reduce MPP$^+$-induced cell death. Previous report has shown that MPP$^+$ up to 2 mM does not cause a significant thiol oxidation in isolated brain mitochondria (Lee et al., 2000). These results hint that the thiol oxidation may not play an important role in the cytotoxic effect of MPP$^+$.

Some antioxidants prevent apoptotic cell death in the dopaminergic cell lines and SH-SY5Y cells treated with MPP$^+$ (Banaclocha et al., 1997; Seaton et al., 1997). Previous studies suggested that SA has stronger antioxidative activity (Zhao et al., 1996). Analysis of molecular structure of SA also indicated that it possesses a potent capacity for scavenging free radicals. The phenolic hydroxyl group in the structure of the SA may be related to its antioxidant activity. Our present results has shown that SA decreases the MPP$^+$-induced accumulation of ROS, and attenuates MPP$^+$-induced mitochondrial damage and cell death in SH-SY5Y cells. Based on these findings, we postulated that the antioxidative properties of SA may contribute to the protection of SH-SY5Y cells from damage by MPP$^+$.

However, some other mechanisms could also be pertinent in the SA protective mechanism. It is increasingly apparent that mitochondria lie at the centre of the cell death regulation process. Induction of apoptosis often converges on the mitochondria to induce permeability transition and release of apoptotic proteins into the cytoplasm resulting in the biochemical and morphological alteration of apoptosis. Although the precise mechanism by which Bcl-2 family members act remain unclear, it has been established that they play a key role in the mitochondrial apoptotic pathway (Cory and Adams, 2002). Bax and Bcl-2, the two main members of this family, influence the permeability of the mitochondrial membrane. Bax is a pore-forming cytoplasmic protein, translocates to the outer mitochondrial membrane, influences its permeability and induces cytochrome c release from the intermembrane space of the mitochondria into the cytosol, subsequently leads to cell death (Cropton, 2000). The anti-apoptotic Bcl-2 is associated with the outer mitochondrial membrane where it stabilizes the membrane permeability, thus preserving mitochondrial integrity, suppressing the release of cytochrome c and inhibiting cell death (Yang et al., 1997). Cell survival in the early phases of apoptotic cascade depends mostly on the balance between the pro- and anti-apoptotic proteins of the Bcl-2 family. In this regard, the Bax/Bcl-2 ratio may better predict the apoptotic fate of the cell than the absolute concentrations of either (Cory and Adams, 2002).

Our present study shows that MPP$^+$ has profound effect on the Bcl-2 family proteins in SH-SY5Y cells. MPP$^+$ upregulates Bax expression, while it does not affect the level of Bcl-2 expression. Consequently, the ratio of the pro-apoptotic Bax to the anti-apoptotic Bcl-2 increases significantly upon treatment with MPP$^+$, which consists with the previous studies (Blum et al., 2001). However, Veech et al. (2000) found that Bcl-2 also increased following MPP$^+$ treatment. Since the Bax/Bcl-2 ratio was high, they hypothesized a compensatory induction of Bcl-2 that was unable to counteract the apoptotic action of Bax, resulting in caspase-3 activation and cell death. Our results showed that treatment with SA reduced the expression of Bax and increased the expression of Bcl-2 significantly, thereby ameliorated the MPP$^+$-induced Bax/Bcl-2 ratio elevation in SH-SY5Y cells. O'Malley et al. (2003) demonstrated that the targeted expression of Bcl-2 attenuated MPP$^+$-induced cell death. Flow cytometry analysis revealed that SA reduced the number of apoptotic cells evoked by MPP$^+$ (Fig. 8). Thus, the effect of SA on MPP$^+$-induced apoptosis may be, at least partly, mediated by regulating the expression of Bax and Bcl-2.

Given the key role of the ratio between Bax and Bcl-2 proteins in the apoptotic cascade, it is not surprising that in our experiments treatment with SA is also associated with the inhibition of the downstream apoptotic signaling pathways, finally preventing activation of caspase-3. The previous studies implicated that mitochondrial dysfunction plays an important role in MPP$^+$-induced cytotoxicity, as a result of the decrease of ATP and mitochondrial permeability transition (Seaton et al., 1997). In this study, the involvement of mitochondria in MPP$^+$-induced apoptosis was investigated by taking into account the loss of mitochondrial membrane potential and the release of cytochrome c. Our results showed that SA prevented MPP$^+$-induced collapse of mitochondrial membrane potential and release of cytochrome c from mitochondria. High levels of mitochondrial membrane potential are necessary to maintain closure of a multi-protein pore and the mitochondria permeability transition pore (Tatton and Olanow, 1999). The opening of the mitochondrial permeability transition pore causes a release of apoptogenic substances such as cytochrome c from mitochondria into cytoplasm (Nicholls and Budd, 2000). Cytochrome c release from mitochondria was proved to play a critical role in apoptosis (Kluck et al., 1997) and has been observed in MPP$^+$-treated cells (Kakimura et al., 2001).

Caspases play an important role in the apoptotic process in two ways (Markus, 2000): the death receptor pathway and the mitochondrial apoptotic pathway. Whichever pathway is involved, caspase-3 acts as an apoptotic executor. Capase-3 activates DNA fragmentation factor, which in turn activates endonucleases to cleave nuclear DNA, and ultimately leads to cell death. In the mitochondrial pathway, a variety of stimuli trigger mitochondrial permeability transition and the release of cytochrome c, and then activating caspase-3. In this study, the observation that treatment with MPP$^+$ leads to an increase in caspase-3 activity is in agreement with

previous studies (Blum et al., 2001), however, a co-treatment with SA effectively suppressed MPP$^+$-induced activation of capase-3 (Fig. 7). SA suppressed MPP$^+$-induced caspase-3 activation, suggesting that SA may act upstream of caspase-3 to block apoptosis. From our own observation, a decrease in caspase-3 activity correlates well with a decrease in the Bax/Bcl-2 ratio, as pro-apoptotic Bax is thought to be upstream of the caspases in the mitochondria-mediated apoptotic death pathway (Adams and Cory, 2001). Anti-oxidant enzymes (SOD and catalase) also suppressed MPP$^+$-induced caspase-3 activation, suggesting that the mechanisms by which SA inhibits MPP$^+$-triggered activation of caspase-3 might include both its antioxidative activity and its regulatory function in Bcl-2 family.

The prospects for developing an anti-apoptotic compound which modifies progression of Parkinson's disease appear favorable. Evidence from both postmortem Parkinson's disease brain tissue and cellular and animal model suggests that pathways involving p53/Bcl-2 family members/mitochondrial membrane permeabilization may represent suitable targets, although death receptor-mediated pathways may also play a role. Our results described that SA decreased Bax/Bcl-2 ratio in MPP$^+$-treated SH-SY5Y cells. The effects of SA presented here resemble that of neuroprotective drugs green tea polyphenol, epigallocatechin-3-gallate (EGCG), and rasagilin, which similarly alter Bcl-2 and Bax expression (Mandel et al., 2003). Based on these reports and the observations in the present study, we hypothesized that SA modulates the Bcl-2 family proteins level in response to MPP$^+$ treatment, and then regulates a succession of mitochondria-mediated downstream molecular events including cytochrome c release and sequential activation of caspase-3. These findings, taken together, support that SA-mediated cytoprotection is due, in part, to inhibition of the mitochondrial apoptotic pathway. Our experiments also show that SA prevents nuclear condensation and reduces the number of apoptotic cells induced by MPP$^+$, which provides further evidences for the anti-apoptotic properties of SA.

In summary, our results show that SA protects SH-SY5Y cells against MPP$^+$-induced cytotoxicity. Its anti-oxidative and anti-apoptotic properties render this natural molecule potentially protective against MPP$^+$-induced cytotoxicity. Further studies of neuroprotective mechanisms of SA in detail are necessary before definite conclusions can be drawn. Based on the protective effects of SA on MPP$^+$-induced cell injury, our results presented in this report may open up a new clinical perspective in progressive neurodegenerative diseases such as Parkinson's disease.

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